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(54) Enhancing keratinocyte migration with clostridiopeptidase-A-collagenase

(57) Clostridiopeptidase A collagenase is used to enhance the migration and proliferation of keratinocytes in wound healing or in the growth of artificial skin grown *in vitro*. The wound is contacted with an effective amount of the purified enzyme that is substantially free from other proteinases. The artificial skin is grown in the presence of an effective concentration of purified Clostridiopeptidase A collagenase substantially free from other proteinases.

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Description

This invention generally relates to the use of a particular form of collagenase to increase the motility of keratinocytes.

Keratinocytes are the predominate cell type of the epidermis. They arise by mitotic division from the stem cells constituting the deepest layer of the epidermis. The keratinocytes migrate upwardly, changing in structure and function until they become mature keratinized cells at the surface of the skin and are eventually sloughed off.

The rate of healing of wounds is affected, inter alia, by the rate of keratinocyte migration and proliferation.

Collagenase has been used to ameliorate various pathologic conditions of the body, and the effect of endogenesis collagenase on certain body functions has been studied.

Chiulli and Wegman, U.S. Patent 3,705,083 (1972), produced from Clostridium histolyticum a combination of collagenase and another protease, and used it in ointment to debride necrotic tissue from dermal lesions such as burns, infected wounds and ulcers. This ointment has been on the market for the past 25 years. They also proposed using the combination as an injectable solution to facilitate internal sloughing and reabsorption of physiologically antagonistic tissue.

Sussman, U.S. Patent 3,678,158 (1972), injected purified collagenase into herniated intravertebral discs.

Cope, U.S. Patent 4,174,389 (1979), used Clostridiopeptidase A collagenase for the selective lysis of collagen fibrils in the vitreous of the eye.

Pinnell, U.S. Patent 4,524,065 (1985), treated mammalian cicatrices such as acne scars, keloids and other hypertrophic scars by intralesional injection of purified collagenase.

Wehling, U.S. Patent 5,173,295 (1992) used purified collagenase to enhance regeneration of injured nerves.

Gelbard, U.S. Patent 4,338,300 (1982), injected collagenase into the plaques of Peyronie's Disease.

W. E. Zimmerman, _____ (pp. 131-141) "The Importance of Collagenase for the Local Treatment of Major Burns," states that collagenase used on burns exerts a concomitant beneficial effect on the formation of tissue proliferations and may thus be used to advantage in the treatment of varying types of wounds.

Herman, Journal of Cardiovascular Pharmacology 22 (Suppl. 4): S25-S36 (1993), "Molecular Mechanisms Regulating the Vascular Endothelial Cell Motile Response to Injury," reported that a commercial non-homogeneous preparation of bacterial collagenase routinely used for the isolation of vascular cells from blood vessel segments increased the rate of migration of vascular endothelial cells on an injured epithelial cell-synthesized matrix in vitro from two to five times the rate for vascular endothelial cells on intact matrix.

The present invention enhances the migration and proliferation of keratinocytes in wound healing by contacting same with Clostridiopeptidase A collagenase (EC 3.4.24.3), obtained by fermentation of Clostridium histolyicum, that has been purified to be substantially free from other proteinases. Preferably, an open wound in the skin is treated by contacting exposed sub-cellular matrix with the said purified collagenase in an amount effective to enhance the rate of migration of keratinocytes towards the wound edges. Contacting keratinocytes means contacting them directly, and/or indirectly by contacting the sub-cellular matrix.

The purified collagenase is preferably applied as an aqueous solution, e.g. dissolved in phosphate-buffered saline. It may also be used in admixture with other pharmaceutically acceptable liquid or solid carriers including slow release carriers. The mature and use of such carrier is within the skill of the art.

Suitable concentrations may range from about 0.5 ABC units collagenase/ml or less up to about 150 ABC units/ml or more, i.e. about 5 µg/ml or less up to about 1,500 µg/ml or more. Concentrations often will be in the range of about 2 to about 50 ABC units/ml. The amount of the purified collagenase applied will be sufficient to increase substantially the migration rate of the keratinocytes towards the wound edges, preferably at least three-fold over the rate that would prevail without the treatment. Of course, the larger the wound the greater the amount of the purified collagenase to be used. Also, the more body fluid present or expected to be present in the wound, the higher the concentration of collagenase solution that will be used. The physician will use his/her professional judgment in these matters.

The potency assay of collagenase is based on the digestion of undenatured collagen (from bovine tendon) at pH 7.2 and 37° C for 20-24 hours. The number of peptide bonds cleaved are measured by reaction with ninhydrin. Amino groups released by a trypsin digestion control are subtracted. One net ABC unit of collagenase will colubilize ninhydrin reactive material equivalent to 1.09 nanomoles of leucine per minute.

The assay of collagenases for other proteinases is based on ability to digest casein. This caseinase assay procedure combines (1) the idea of Reimerdes and Klostermeyer [methods Enzymol 45: 26-28 (1976)] to determine the amount of primary amino groups present int he trichloroacetic acid-soluble digestion products with (2) the method of Udenfriend et al. [Science 178: 871-2 (1972)] to detect the primary amino groups fluorometrically. The sample is incubated with added casein, which is not soluble, at 37°C for 20-22 hours. The sample is quenched with trichloroacetic acid and the undigested casein is then centrifuged out. Solubilized peptides result from the action of caseinase in the sample on the added casein. Each peptide molecule has a terminal primary amine group. Fluorescamine™ is added to the supernatant and reacts with primary amine groups producing fluorescent molecules. The fluorescence is meas-

ured and a calculation gives a caseinase activity as FFC units.

The present invention in another aspect provides a method of enhancing the migration and proliferation of keratinocytes in the growth of artificial skin in vitro by growing the artificial skin in the presence of added Clostridiopeptidase A collagenase that has been purified to be substantially free from other proteinases. Artificial skins are useful as temporary skin grafts for burns and ulcers, and are used for testing of cosmetics and household cleansers in vitro.

In applying the present invention, the purified Clostridiopeptidase A collagenase described herein is added to provide a concentration in the growth medium of from about 0.5 ABC units collagenase/ml or less to about 150 ABC units/ml or more, thereby enhancing the migration and proliferation of keratinocytes. Some examples of artificial skins whose growths can be favorably affected by this invention follow.

Advanced Tissue Sciences of La Jolla, CA has marketed Dermagraft™ as a skin substitute. A mesh scaffold made from lactic acid-glycolic acid copolymer, about 90 µm thick with openings of about 200-220 µm was seeded with skin fibroblasts from neonatal foreskins. The cells bridge sufficiently to secrete skin proteins and proteoglycans. See Hubbell, JA et al. Chemical and Engineering News pp. 42- 53 (March 13, 1995).

Graftskin™ has been introduced by Organogenesis of Canton, MA. See Nolte CJ et al. Journal of Anatomy 185 (Pt. 2): 325-33 (1944 Oct.)

Advanced Tissue Sciences has also marketed Skin^2 ™ as a skin substitute for in vitro testing of cosmetics, household chemicals and other products. See Stoppie P et al. European Journal of Morphology 31 (1-2): 26-9 (1993).

See also Hansbrough JF et al., Journal of Burn Care & Rehabilitation 14(5): 485-94 (1993).

EXPERIMENTAL

The effect of collagenases of varying purities on the motility and proliferation of keratinocytes was determined in vitro, employing sub-cellular matrices synthesized from vascular endothelial cells.

Vascular endothelial cell culture

Endothelial cells are isolated from living bovine vessel segments. Rings of aortae are obtained on ice from an abattoir sutured at the ends and filled with balanced salts (BSS). Endothelial cells are released from the intima using 0.1% collagenase dissolved in BSS by incubation at 37°C for 30 minutes-one hour. Cells are pelleted at 200g for 5 minutes at room temperature and the resultant pellet resuspended in growth media containing 5% calf serum. Cells are plated into tissue culture at 50K cells/25 cm^2. Following growth to confluence, cells are typsinized and passaged at 1:5 split. Cells are used between passages 5- 15.

Endothelial-derived matrix:

One week post-confluent endothelial cells are washed with BSS prior to lysis in sterile solution containing 0.5% sodium deoxycholate in 0.015M MaCl, 0.001M EGTA buffered with 0.02M Tris-Cl, pH 7.8 with 0.001M phenyl methyl sulfonyl fluoride (PMSF) as a protease inhibitor. Two room temperature detergent treatments, each lasting 15 minutes, are followed by five washes with BSS, each wash lasting 5 minutes. Keratinocytes are then plated directly onto washed matrices or matrices are digested with collagenase solutions.

Treating matrices with collagenases:

Endothelial matrices, prepared as described above are treated for 60 minutes at 37°C with various preparations of collagenases dissolved in BSS (0.9% sodium chloride) containing 2mM $CaCl_2$. Collagenase dose ranges from 0-128 U/ml; IU/ml = 10µg/ml collagenase. Matrices treated with the enzyme are then washed with BSS without calcium and keratinocytes and then plated. (U means ABC units).

Human keratinocytes:

At circumcision, foreskins are placed into GIBCO Keratinocyte-SFM containing Gentamycin (Cat.nos. 17005-018 and 157-015) at 5 µg/ml. Tissue is then rinsed in BSS with gentamycin prior to cutting into pieces of 3-4 mm^2. Tissue pieces are then incubated for 18 hrs at 4°C in 25U/ml dispase (Collaborative Research cat.no. 40235). After dispase incubation, the epidermal layer of human keratinocytes is lifted from the dermis and placed into 15 ml centrifuge tubes containing trypsin-EDTA (2ml). Following a 15 minute incubation at 37°C, cells are sedimented and plated in Keratinocyte-SFM at an initial seeding density of 3 x 10^6 cells/T (75cm^2) flask. cells are incubated and passaged using trypsin-EDTA when the flask is 60-70% confluent.

Motility and growth studies:

For cell motility (wound healing) studies, keratinocytes are plated at near-confluent densities on intact or collagenase-treated matrices (100K cells/cm^2). Cells plated on glass microscope cover slides with matrices attached are then placed into a specially-designed culture chamber that mounts on the stage of an inverted, interference or phase contrast light microscope. Cells are warmed to 37°C while viewed using video-enhanced optics coupled with computer-assisted imaging work station and software developed in the lab to automatically track living cell migration (Cell Tracker, Askey and Herman, 1988; Computers and Biomedical Res. 21:551-61). Keratinocytes bordering artificially created wounds made with fire-polished pasteur pipets, or keratinocytes at the edge of intact sheets, are then recorded for motility as a function of matrix condition.

For cell proliferation studies, keratinocytes are plated in triplicate onto plastic or matrix (intact or collagenase treated; doses from 0-64 U/ml, with 4 U/ml sufficient to deliver maximal proliferative responses seen within 7 days post-plating) at 2-5K cells/cm^2. Cells are fed on alternate days with Keratinocyte-SFM and triplicate wells of cells counted directly using a Coulter Counter, ZF. Cell counts, together with errors of the mean are plotted as a function of time and condition using Kaleidograph, a software support compatible with the MacIntosh computer workstation in the lab.

Crude Collagenase:

This was obtained substantially as described by Chiulli and Wegman in U.S. 3,705,083 (see page 1 above), with minor modifications. It is the powder used as the active ingredient in Santyl^R Ointment. The collagenase content ranges from 100-300 ABC units per mg, and the proteanase content ranges from 30 to 240 FFC units/mg.

Cleaned-Up Collagenase:

Crude Collagenase was suspended in distilled water and after thorough stirring was centrifuged. The centrifuge tubing were decanted and the supernatant was again centrifuged. The resultant clarified solution was "cleaned up" product.

ABC Purified Collagenase:

This was prepared from crude collagenase by chromatography substantially eliminating other proteinases. The purified collagenase used contained only about 0.1 FFC units proteinases per mg.

Pool 3A

This was a combination of fractions discarded in the chromatography yielding Purified Collagenase.

Clostripain

A proteinase present in crude. This sample was a commercially available clostripain.

The collagenases were provided by Advance Biofactures Corporation of Lynbrook, NY 11563.

In the following tests, the concentration of collagenase used in all of the samples of varying purities was 4 ABC units per ml.

Figure I presents graphically the motility (migration) data.

Figure II presents graphically the proliferation data.

Table I gives the migration results in terms of Migration Index.

Table II gives the proliferation results in terms of Proliferation Index.

TABLE I

MATRIX MODULATES KERATINOCYTE SHEET MIGRATION	
Matrix	Migration Index (MI)$
Untreated	1.0
ABC Purified Collagenase	3.1

$$\$MI = \frac{\text{mean motility (experiment)}}{\text{mean motility (control)}}$$

TABLE I (continued)

MATRIX MODULATES KERATINOCYTE SHEET MIGRATION	
Matrix	Migration Index (MI)$
Cleaned-Up Crude Collagenase	2.4
Crude collagenase	1.9

$MI = \frac{\text{mean motility (experiment)}}{\text{mean motility (control)}}$

TABLE II

MATRIX MODULATION OF KERATINOCYTE PROLIFERATION	
Matrix	Proliferation Index (PI)$
Untreated	1.0
ABC Purified Collagenase	2.1
Cleaned-Up Crude Collagenase	1.4
Crude Collagenase	1.3
Clostripain	1.3
Pool 3A	1.3

$PI = \frac{\text{mean cell counts (experiments)}}{\text{mean cell counts (control)}}$

In these tests, treatment of the extracellular matrix with the purified collagenase potentiated keratinocyte migration 3-fold over the untreated matrix control, and potentiated keratinocyte proliferation 2-fold over the untreated matrix control. Other similar tests gave migration rates up to 10-fold over untreated matrix. Further in every instance the results with the purified collagenase were superior to those obtained with the less pure (cleaned up crude and crude) collagenases.

Another series of tests employed three kinds of synthetic sub-cellular matrices, prepared respectively from normal skin fibroblasts, endothelial cells, and cells from keloid scars. Each was treated with concentrations of purified collagenase ranging from 1 ABC unit/ml to 64 ABC units/ml, and the rate of cell growth (proliferation) is measured. With each matrix the growth rate at 64 units/ml was taken as the rate beyond which a higher dosage would have only limited effect. The dosage giving 50% of that growth rate (designated ED 50) was for each matrix about 1 ABC unit/ml.

Claims

1. A method of growing artificial skin *in vitro,* wherein the migration and proliferation of keratinocytes is enhanced by growing said artificial skin in the presence of an effective concentration of added purified Clostridiopeptidase A collagenase substantially free from other proteinases.

2. A method according to claim 1 wherein the concentration of said collagenase in the growth medium is from 0.5 ABC units/ml to 150 ABC units/ml.

3. A method according to claim 2 wherein the collagenase concentration is within the range of 2 to 50 ABC units/ml.

4. The use of purified Clostridiopeptidase A collagenase substantially free from other proteinases in the preparation of a medicament for enhancing the migration and proliferation of keratinocytes in wound healing.

5. The use of purified Clostridiopeptidase A collagenase substantially free from other proteinases in the preparation of a medicament for treating an open wound in the skin by contacting exposed sub-cellular matrix with said medicament to enhance the rate of migration of keratinocytes towards the wound edges.

6. Use according to claim 4 or claim 5 wherein said medicament is prepared in the form of an aqueous solution

containing from 0.5 ABC units collagenase/ml to 150 ABC units/ml.

7. Use according to claim 6 wherein the collagenase concentration in said medicament is within the range of 2 to 50 ABC units/ml.

FIG. 1

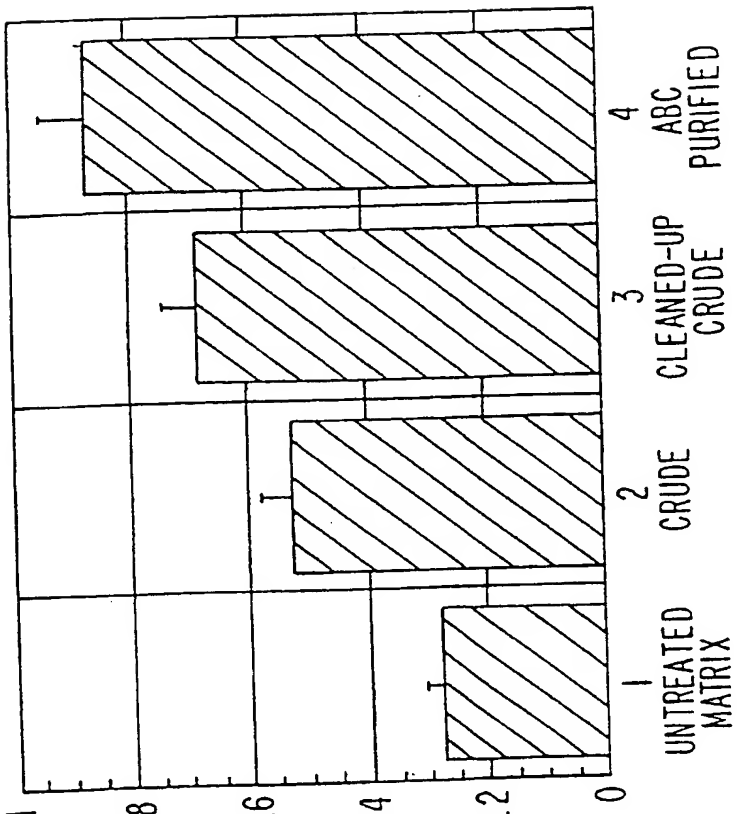
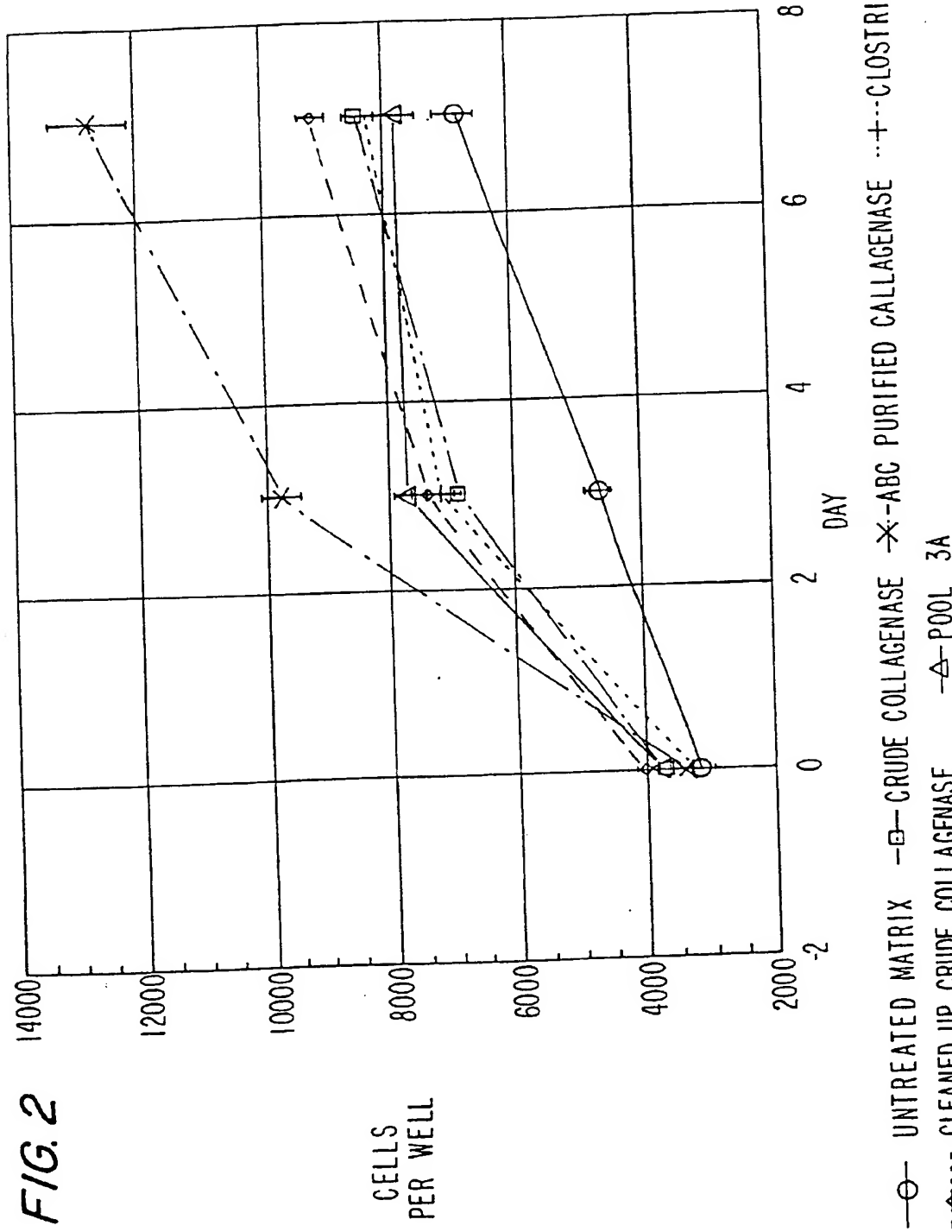

AVERAGE CELL VELOCITY (MICRONS/MIN)
1
0.8
0.6
0.4
0.2
0
1 UNTREATED MATRIX
2 CRUDE
3 CLEANED-UP CRUDE
4 ABC PURIFIED

FIG. 2


CELLS PER WELL
14000
12000
10000
8000
6000
4000
2000
-2
0
2
4
6
8
DAY
UNTREATED MATRIX
CRUDE COLLAGENASE
ABC PURIFIED CALLAGENASE
CLOSTRIPAIN
CLEANED UP CRUDE COLLAGENASE
POOL 3A

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 30 3921

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	ZIMMERMANN, W.E. ET AL.: "Der Kollagenase-Effekt ..." RES. EXP. MED., vol. 157, 1972, pages 244-246, XP002097652 * p. 245,"Ergebnisse" *	4-7	C12N5/00 A61K38/48 C12N5/06 C12N9/52
X	EP 0 543 521 A (ADVANCE BIOFACTURES OF CURACAO N.V.) 26 May 1993 * claims 1 and 15 *	4,5	
A	HANSBROUGH, J.F. ET AL.: "Composite grafts of human keratinocytes ..." J. BURN CARE REHABIL., vol. 14, 1993, pages 485-494, XP002097653 * abstract; p. 486, section "Preparation of composite grafts" *	1-3	
A	"Collagenase" SIGMA CATALOGUE,1995, page 279 XP002097654 * Section "High-purity: Chromatographically Purified" *	1-7	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
C12N
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	24 March 1999	HERMANN R.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 96 30 3921

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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24-03-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 543521 A	26-05-1993	US 5393792 A	28-02-1995
		AU 654881 B	24-11-1994
		AU 2853592 A	27-05-1993
		CA 2083333 A	21-05-1993
		JP 7089871 A	04-04-1995
		US 5422103 A	06-06-1995
		US 5514370 A	07-05-1995

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

US005474770A

United States Patent [19]

Broly et al.

[11] Patent Number: 5,474,770

[45] Date of Patent: Dec. 12, 1995

[54] BIOLOGICAL SUPPORT FOR CELL CULTURES CONSTITUTED BY PLASMA PROTEINS COAGULATED BY THROMBIN, ITS USE IN THE PREPARATION OF KERATOCYTE CULTURES, THEIR RECOVERY AND THEIR TRANSPORT FOR THERAPEUTIC PURPOSES

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[73] Assignee: **Centre Regional de Transfusion Sanguine de Lille**, Lille, France

[21] Appl. No.: **850,260**

[22] Filed: **Mar. 12, 1992**

Related U.S. Application Data

[63] Continuation of Ser. No. 444,967, Dec. 4, 1989, abandoned.

[30] **Foreign Application Priority Data**

Dec. 6, 1988 [FR] France 88 15950

[51] **Int. Cl.**[6] **C12N 5/08**; C07K 14/745; A01N 1/02; A61F 2/10

[52] **U.S. Cl.** **424/94.64**; 424/530; 435/240.21; 435/240.243; 435/1.1; 435/214; 514/12; 530/381; 530/382; 623/15

[58] **Field of Search** 435/240.23, 240.21, 435/240.243, 1, 214; 530/381, 382; 623/15; 424/530, 94.64; 514/12

[56] **References Cited**

U.S. PATENT DOCUMENTS

2,469,193	5/1949	Cohn	530/831
2,533,004	12/1950	Ferry et al.	530/381
3,920,625	11/1975	Andersson et al.	530/831
4,414,976	11/1983	Schwarz et al.	530/381
4,427,650	1/1984	Stroetmann	530/381
4,442,655	4/1984	Stroetmann	530/381
4,452,893	6/1984	Ng et al.	435/240.3
4,485,096	11/1984	Bell	623/1
5,260,420	11/1993	Burnout-Radosevich et al.	530/382

FOREIGN PATENT DOCUMENTS

0085923	1/1983	European Pat. Off. .
0143648	11/1984	European Pat. Off. .
0305243	7/1988	European Pat. Off. .

OTHER PUBLICATIONS

Nuzzolo, et al. Tissue Culture Techniques pp. 84–85 Warren H. Green, Inc. St. Louis, Mo. 1983.

Hunyadi, et al. Keratinocyte Transplantation: Covering of Skin Defects by Autologous Keratinocytes.

Biological Abstracts vol. 85 No. 5 Abstract 43762 Mar., 1988.

Cohn et al. Preparation and Properties of Serum and Plasma Proteins IV. J. American Chemical Society vol. 68 pp. 459–475 Mar., 1946.

Search Report from FR 88 15950.

Ronfard et al., "Use of human keratinocytes . . . treatment of burn wounds," *Burns*, (1991) vol. 17, No. 3, pp. 181–184.

Primary Examiner—Margaret Moskowitz Parr
Assistant Examiner—Ron Schwadron
Attorney, Agent, or Firm—Birch, Stewart, Kolasch & Birch

[57] **ABSTRACT**

The present invention relates to a biological support for cell cultures formed by the coagulated mixture of a concentrate of plasma proteins and thrombin.

The protein concentrate is obtained by precipitating fresh plasma with ethanol and contains balanced proportions of fibrinogen, Factor XIII and fibronectin. The thrombin concentration is adjusted to obtain the desired consistency of the support coagulated in the form of a film.

The biological support is preferably used for preparing a culture of keratinocytes, recovering them in the form of a reconstituted tissue and transporting same. The reconstituted tissue is thus particularly suitable for use as a graft.

16 Claims, No Drawings

BIOLOGICAL SUPPORT FOR CELL CULTURES CONSTITUTED BY PLASMA PROTEINS COAGULATED BY THROMBIN, ITS USE IN THE PREPARATION OF KERATOCYTE CULTURES, THEIR RECOVERY AND THEIR TRANSPORT FOR THERAPEUTIC PURPOSES

This application is a continuation of application Ser. No. 07/444,967 filed on Dec. 4, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a biological support for cell cultures, constituted by a coagulated mixture of a concentrate of plasma proteins and thrombin, its use in the preparation of keratinocyte cultures and their transport in the form of reconstituted epidermises, and their use for therapeutic purposes.

2. Description of Related Art

The reconstitution in a laboratory of a living skin similar to the human skin from a few cells obtained from a biopsy, or of a simplified skin performing the physiological functions of a normal skin, is being studied extensively In the aim of replacing skin damaged by a serious disease (genetic, etc.) or destroyed by major burns.

The skin is a complex organ composed of three juxtaposed tissues: the epidermis, 85% of which is constituted by keratinocytes which form the impermeable horny layer that isolates the body from the outside environment; the dermis, which comprises cells, including fibrocytes, separated by a connective tissue composed mainly of collagen; the dermis lies on the hypoderma, which includes the cells dedicated to storing fats. Artificial reconstitution of such a complex organ thus poses numerous problems.

The first tissue to have been partially reconstituted in vitro was the dermis, this being achieved by the Bell team (Bell et al.) *Proc. Natl. Aced. Sci.* 76-1979-1274).

Starting with skin biopsies, fibroblasts have been successfully established in cultures, first in monolayers, then, after a number of passages, by dispersing these cells in culture medium containing collagen (extracted from rat's tail tendons), the latter forming a gel and permitting three-dimensional cultures. In such cultures, the fibroblasts can be seen interacting with the matrix of the collagen, organizing it and contracting It as in a normal dermis. This tissue, reconstituted in vitro, is known as an "equivalent dermis". After a few weeks' growth, the mechanical qualities of the equivalent dermis allow it to be used for grafting onto a patient or injured person. It does not appear to be rejected by its host. However, this equivalent dermis is merely a temporary dressing: it cannot restore the the cutaneous barrier function.

Furthermore, the Green team (H. Green et al. Proc. Natl. Aced. Sci. 76, 1979, 5665) has developed a method and a culture medium enabling keratinocytes to be grown for long periods. This method includes inoculating the keratinocytes dispersed with trypsin on a pre-established monolayer of fibroblasts, in particular 3T3 cells, lethally irradiated and which serve as a nutritive layer and as a matrix. The epidermal layer develops very rapidly to form a tissue having a thickness of 3 to 5 cells; it can be grafted onto a patient and continue to differentiate in situ. It has already proved possible to save those suffering from severe burns using this technique (G. Gallico et al. New England J. Med. 311, 1984, 448).

With Green's technique, it is possible to obtain, from a biopsy of two square centimeters an epidermis of one square meter in the space of three weeks.

Recovery of the reconstituted tissue in order to make a graft therefrom still poses a number of technical problems. It is, indeed, necessary to detach the cells from the culture dish, using an enzyme treatment, without dissociating them from one another; during this operation, one always observes a retraction of the cell layer, hence a loss of a certain percentage of the surface area of the graft. Once the reconstituted tissue has been detached, it has to be fastened to a support that enables it to be transported and grafted onto the patient. A vaseline treated gauze dressing is generally used. All these manipulations are delicate and time consuming.

It would thus be highly beneficial to have at one's disposal novel biological supports that can be resorbed in time by the patient who has received the graft and which simplify the handling of the cells. In addition, to ensure their availability, these supports or their constituents would have to lend themselves to preparation and packaging in accordance with industrial processes.

SUMMARY OF THE INVENTION

The Applicant has thus developed a biological support for cell cultures constituted by a mixture of a concentrate of plasma proteins that can be coagulated by thrombin and of the quantity of calcic thrombin that is necessary to activate coagulation.

The coagulation of the plasma proteins in the presence of thrombin is chiefly due to the formation of a polymerized fibrin network which imitates the formation of a blood clot. To form a support suitable for the preparation of cell cultures, coagulation is carried out under conditions conducive to the formation of a film and, more particularly, in Petri flasks or in any flask suitable for cell cultures.

DETAILED DESCRIPTION OF THE INVENTION

The concentrate of plasma proteins has already been described by the Applicant in European patent application 88 401 961 3: it is obtained by precipitating fresh plasma, in two successive treatments using a 10% ethanol solution at 4° C. The concentrate contains over 90% of fibrinogen and, per gram of proteins, at least 0.1 IU of Factor XIII and from 0.03 to 0.1 grams of fibronectin. The concentrate is packaged and freeze dried to preserve It until it is used.

The present invention thus also concerns the concentrate of proteins that can be coagulated by thrombin, specially packaged for preparation of the biological support for cell cultures.

At its time of use, the concentrate is redissolved in a saline aqueous solution or in a solution containing a polyvalent protease inhibitor, preferably aprotinin, at a concentration of 3000 KIU/ml.

To activate the coagulation process, hence the formation of the film serving as a support for the cells, thrombin is added, with or without calcium. The process includes the transformation of fibrinogen into fibrin through the action of thrombin and the polymerization of monomeric fibrin with fibronectin through the action of Factor XIII activated by Ca^{++} ions.

To form the support according to the invention, which is particularly appropriate for cell cultures, the thrombin con-

centration is preferably adjusted to approximately 10 IU/ml (a far lower concentration than the one used when the desired consistency is different, as in the case of biological glues— patent No. 88 401 961 3, mentioned above).

According to different forms of embodiment of the invention, it is possible to incorporate In the support various additives particularly designed to promote cell multiplication in vitro or in situ and thus favoring the healing of the wound after grafting.

The support can thus contain an additive promoting cell multiplication such as a growth factor and, more particularly, EGF ("epidermal growth factor").

A healing agent or an antibiotic can also be incorporated.

The support according to the invention is particularly advantageous when preparing human keratinocyte cultures. These cells can be either primary cultures derived from skin biopsies obtained from a patient and that have undergone between 1 and 4 passages in 1/15 to 1/20 dilutions, or cells preserved in the form of banks in liquid nitrogen.

These keratinocytes established in a confluent layer are trypsinized and replaced in suspension in an appropriate culture medium at the time of their seeding on the support according to the invention.

The use of the biological support according to the invention can be adapted in three different ways.

According to a first method of use, the biological support is prepared in the form of a film, by mixing its two constituents in a culture dish; a suspension of keratinocytes is seeded on this film, in an appropriate culture medium. When the keratinocyte culture has become confluent or semi-confluent, it forms a replacement tissue that can be recovered directly as a graft that can be detached using forceps and transported from the dish to the patient, on whom it is applied as it is, without there being any need for a temporary support such as gauze. This makes for a considerable saving in working time and 100% recovery of the tissue grown.

According to another method of using the support according to the invention, the two constituents of the support are mixed with the keratinocytes suspension in such a way as to integrate the cells in the film that will be formed subsequently. According to this method, the two constituents can be mixed with the cell suspension in a culture dish and then used as a graft, as in the method described above; It can also be carried out directly on the patient's wound, prepared to receive a graft, in particular by spraying the biological support and the cells using a vector gas (nitrogen) at a pressure of 2 to 2.5 bars.

According to another method of using the support according to the invention, its two constituents are mixed on a cell layer of keratinocytes pre-established In a culture dish, in such a way that the cells are coated with the film that has formed and can thus be detached and transported in order to be applied as a graft.

The following examples serve to illustrate the invention without thereby restricting its scope.

Example I—Preparation of the biological support for cell cultures

A biological support for cell cultures is prepared by mixing a concentrate of coagulable plasma proteins and the quantity of calcic thrombin necessary to activate coagulation.

A. Preparation of the concentrate of plasma proteins

The preparation of the protein concentrate has already been described by the Applicant in European patent application No. 88 401 961 3. To summarize, use is made of non cryoprecipitated human plasma; it is precipitated twice in succession in a 10% solution of ethanol at a pH of 7.2 and a temperature of 4° C. Between two successive precipitations, the product undergoes a virus inactivation treatment. The precipitate, separated from the supernatant by centrifuging, is washed in ethanol at 4° C. and re-centrifuged. The precipitate is replaced in suspension in a Tris/citrate buffer, adjusted to a protein concentration of approximately 35 g/l and lysine is added thereto at a final concentration of 0.1 to 0.2 g per gram of proteins. After diafiltration to remove the alcohol and the citrate, and to adjust the ionic force, the concentrate is packaged in flasks and freeze dried.

This protein concentrate contains, per gram of proteins, at least 0.9 g of fibrinogen, 0.03 to 0.06 g of fibronectin and 0.15 to 0.30 IU of Factor XIII.

B. Preparation of the support for cell cultures

The protein concentrate described above is replaced in suspension in an aqueous solution, with or without aprotinin, at a concentration of 3000 KIU/ml (kallikrein inhibitor units/ml).

This solution is mixed with an equal volume of calcic thrombin at 10 IU/ml.

For a Petri dish, with a diameter of 10 cm, use is made of 2 ml of protein suspension and 2 ml of thrombin, these two solutions being injected simultaneously using two syringes interconnected by a mixing coupling. The Petri dish is shaken to obtain uniform distribution and the preparation is then allowed to rest for 15 to 20 minutes. It forms a film that covers the dish.

The culture dishes are of the type "non treated for cell cultures", which ensures that the support does not adhere permanently, thus facilitating its subsequent recovery.

This film is then covered with cell culture medium. This medium is renewed several times until the osmotic pressure of the film is stabilized within a range compatible with the physiology of the cells, i.e. between 260 and 340 mosM (milliosmoles).

Alternatively, the reconstituted protein concentrate can be dialyzed before it is mixed with the thrombin.

Example 2—Preparing a keratinocytes culture on the biological support

Use is made of primary cultures of keratinocytes prepared using Green's classical technique from skin biopsies obtained from a patient's skin (or an embryo's skin to form foetal cell banks). These primary cultures can undergo 4 to 5 passages in 1/10 dilution.

A layer of confluent keratinocytes is trypsinized, replaced in suspension in culture medium and seeded in 1/10 dilution on a Petri dish covered with a film of the biological support described in example 1.

After a few hours, the cells adhere to the support, where they then multiply normally until they form a fragment of confluent epidermis having a thickness of 3 or 4 cell layers.

This fragment of reconstituted epidermis, adhering to the support, can be detached from the culture dish using forceps and applied as it is to a wound prepared to receive a graft.

As the cells adhere to the support, there is no need to attach the reconstituted epidermis to another support such as the vaseline treated gauze which has to be used with the other types of culture. This makes for a considerable saving in working time, it being possible to handle 40 Petri dishes

an hour as opposed to the 4 Petri dishes of the conventional techniques.

Furthermore, this support stands up well to handling and does not retract at the time of detachment, which makes it possible to recover 100% of the surface area of the cell layer of the culture,

Example 3—Recovery of a pre-established cell layer using the biological support

Keratinocytes are inoculated according to Green's conventional method, in a Petri dish covered with a layer of lethally irradiated fibroblasts.

When the sheet of keratinocytes is confluent and formed of several layers of cells, the culture medium is removed, an EDTA solution is added for 1 hour 30 minutes, this being followed by washing twice with PBS. The biological support is then poured directly onto the layer of cells, in accordance with the method described in example 2.

When the film is formed over the cells, it can be detached using forceps and used as a graft, as in the preceding example.

Example 4—Incorporation of the cells into the biological support

A syringe of protein solution and a syringe of thrombin containing the keratinocytes in suspension are prepared. These keratinocytes can be taken from a fresh, trypsinized culture or from a bank of cells preserved in liquid nitrogen.

The two syringes are interconnected by means of a mixing coupling and the support containing the cells is sprayed onto the Petri dish (or onto the wound to receive the graft); the cells are thus held in the film during its coagulation. The spraying can be carried out using a vector gas (nitrogen at a pressure of 2 to 2.5 bars).

This spraying does not denature the cells and the cell layer can be observed to reform, in culture in vitro. The cells should thus multiply normally or practically normally, when the mixture is sprayed, in a very thin layer, directly onto a wound.

We claim:

1. A biological support for skin grafts, which comprises approximately 10 IU/ml of calcic thrombin and a mixture of a concentrate of proteins that can be coagulated by thrombin, obtained by treating noncryoprecipitated plasma with ethanol and containing proportions of coagulable fibrinogen, Factor XIII, and plasma fibronectin sufficient to form a biological support in the presence of calcic thrombin.

2. The biological support according to claim 1, wherein the concentrate of coagulable plasma proteins contains over 90% of fibrinogen and, per gram of proteins, and at least 0.1 IU of Factor XIII and 0.03 to 0.1 grams of fibronectin per gram of protein.

3. The biological support according to claim 2, wherein the protein concentrate is obtained by precipitating fresh plasma, in two successive treatments with a 10% solution of ethanol at 4° C.

4. The biological support according to claim 1, wherein the protein concentrate is freeze dried.

5. The biological support according to any one of claims 1 to 3, wherein the protein concentrate is placed in suspension in an aprotinin solution, said aprotinin solution having a concentration of 3000 KIU/ml.

6. The biological support according to any one of claims 1 to 3, wherein it contains, as an additive, an enhancer of cell multiplication.

7. The biological support according to any one of claims 1 to 3, wherein it contains, as an additive, an antibiotic.

8. A method of using the biological support according to any one of claims 1 to 3, which comprises preparing a culture of human keratinocytes, fetal or adult, on said biological support to form a skin replacement tissue and recovering, transporting and applying said skin replacement tissue as a graft.

9. The method according to claim 8, wherein said protein concentrate and calcic thrombin are mixed in such a way as to form a uniform film in a culture dish and the keratinocytes in suspension in culture medium are seeded on said film.

10. The method according to claim 8, wherein said protein concentrate and calcic thrombin are mixed with a suspension of keratinocytes in such a way as to integrate the cells into the film subsequently formed.

11. The method according to claim 9, wherein the keratinocytes suspension is obtained after dispersion of a fresh, pre-established cell layer.

12. The method according to claim 9, wherein the keratinocytes suspension is obtained from a bank of cells preserved in liquid nitrogen.

13. A method of using a biological support according to any one of claims 1 to 3, which comprises recovering a culture of human keratinocytes, fetal or adult, pre-established on the biological support of any one of claims 1 to 3, and transporting said culture and biological support to a patient in need thereof.

14. The method according to claim 13, wherein said protein concentrate and calcic thrombin are mixed on the pro-established cell layer in a culture dish.

15. The biological support according to claim 1, wherein said concentrate contains more than 90% fibrinogen per gram of protein.

16. A composition which comprises a culture of human keratinocytes attached to said biological support of any one of claims 1 to 3.

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